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PRINCIPAL INVESTIGATOR: Sonia Kamrani

CONTRACTING ORGANIZATION: University of Texas M.D. Anderson Cancer Center
Houston, TX 77030

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Sonia Kamrani

E-Mail: skamrani@mdanderson.org

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14. ABSTRACT The roles of estrogen and estrogen receptor (ER) in normal mammary gland development and in transformation to a tumor phenotype have been extensively studied. However, although the cumulative data suggests an important role for ER in breast cancer, the function of ER in regulating cell proliferation in normal and tumor tissue remains unclear. This study investigates the role of ER-alpha on the progression of the cells through different phases of the cell cycle in the presence and absence of its ligand, estradiol. The cellular localization of ER-alpha is also examined during cell cycle transition in MCF-7 cells. Experimental Design: The ER negative MDA-MB 231 and ER positive MCF-7 cell lines were used for study. Cells were synchronized in G1 phase of the cell cycle with lovastatin for 36 hours in estrogen free media and subsequently released from arrest with mevalonate. Transient transfection of MDA MB 231 cells with ER-alpha as well as addition of 17-β estradiol to the cells were performed at the time of release from cell cycle arrest. Cells were harvested at different time intervals (0-68 hrs) after the release and subjected to western blot and flow cytometry analysis. For immunofluorescent detection of ER-alpha MCF-7 cells cultured on coverslips, synchronized with lovastatin and stained with anti-ER alpha antibody. Cells stained to look at ER-alpha in the cell. Results: Synchronization of MCF-7 cells revealed that the endogenous ER is subject to cell cycle regulation with its levels peaking at the S/G2 phase of the cell cycle. Similarly, MDA-MB231 cells transiently transfected with ER-alpha demonstrate tight cell cycle periodicity similar to that seen with cyclin B. Specifically, ER-alpha is up-regulated during late S phase and early G2 phase, and is down regulated during late G2 and M phases. In contrast, in non-synchronized MDA-MB 231 cells, exogenously transfected, ER levels first appear within only 12 hours of transfection, and its expression persists for several days. Additionally when 17-β estradiol is added to synchronized populations of MCF-7 cells (endogenous ER expression) or MDA-MB231 cells (exogenous ER expression), there is a significant change on the doubling time of the cells compared to unliganded cells. This change is the result of shortening of the S plus G2/M phases of the cell cycle in the presence of ligand. Our results collectively show that unliganded ER inhibits cell cycle progression during S and G2/M phases in breast cancer cells, which may explain the growth inhibitory role of ER reported in the literature while liganded ER speeds up the exit from G2/M phase of the cell cycle. Lastly, the result of immunofluorescent study shows a translocation of ER-alpha from the cytoplasm to the nucleus during the progression of cell cycle from G1 to G2/M. Conclusion: We conclude that the ER-alpha expression under non-liganded and liganded conditions have opposing effects on the progression of the cell cycle in G2/M phase.

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Cell cycle regulatory roles of ER-alpha in breast cancer

Introduction:

The human ER belongs to nuclear hormone receptors, which are a family of hormone-activated transcription factors that can initiate or enhance the transcription of genes containing specific hormone response elements. Estrogen diffuses through the plasma membrane of cells where it binds to the ER (1). First it was thought that estrogen binds to the ER in the cytoplasm and then translocate in to the nucleus, however the current dogma is that ER is a nuclear transcription factor that initially interacts with estrogen inside the nucleus (2).
Like all steroid hormone receptors, ligand-free ER-α is sequestered in an inactive form associated in a large molecular complex organized around the heat shock protein of 90 kDa (hsp90) (3). Hsp90 proteins are stabilized when integrated in the molecular chaperone complex. Inhibition of hsp90's ATPase activity by some of the ligands, targets the substrate to ubiquitination and its 26S-proteasome-mediated degradation (4, 5). Once estrogen binds to the ER, heat shock proteins dissociate and a change in conformation and homodimerization in the ER protein occurs. These events trigger an estrogenic response in the cell (1). Estrogenic response consists of three to four fold increase in the basal level of ER phosphorylation upon treatment with estrogen and antiestrogens. (6)

Moreover, several recent reports have shown that cyclins, which are expressed in a cell cycle-dependent manner, can regulate steroid receptor function and that this regulation is independent of the kinase partner (7, 8, 9, 10). There are some studies on the translocation and function of PR, which is dependent on the cyclinA and is cell cycle dependent. In breast cancer cells expressing the progesterone receptor (PR), progesterone induces a biphasic change in cell cycle progression, initially accelerating the cells to progress through the cell cycle and then inducing an arrest in the G0/G1 phase of the subsequent cycle. (8). All of these data on the members of nuclear receptor super families led us to measure the activity of ER as a function of cell cycle in MCF-7 breast cancer cells, which express endogenous ER-α and in MDA-MB231 cells, which express exogenous adenovirally expressed ER-α.

However, the key to elucidating the mechanism of estrogen action is the identification of the cell cycle phase that has the highest expression and activity of ER.
Identification of interacting proteins, specific for each phase of the cell cycle is the next important step to shed light on the mechanism underlying the different ER actions through the cell cycle phases.

Body:

Task 1in the proposed study is to determine the mechanism of cell cycle regulation of ER-α in breast cancer. For this step we stated four steps.

a. Generating stable clones of MDA-MB231 cells overexpressing ER-α.

Due to the problems in the generation of stable clones of MDA-MB231 cells to express ER-α, we changed the strategy of making stable clones. We have started to construct the Adenoviral vectors of ER-α to accomplish this step. Adenoviral expression of ER-α in the ER-α negative cells is an efficient method to express high levels of exogenous ER-α and is a good substitution for the stable clones.
Adenoviral vector expressing ER-α is necessary for the subsequent studies of the effect of the exogenous ER-α on the cell cycle in the other wise ER- negative cells.
We have made the construct of Adenovirus containing ER and also Adenovirus containing GFP-ER. We are in the process of amplification and purification of the virus for the subsequent studies.

b. Use of different methods of synchronization to monitor cell cycle regulation of ER-α.

We have been optimizing Nocodazol synchronization on MCF-7 cells. We have not been able to find a proper concentration and timing that synchronize most of the cells in G2/M and to be reversible. The results of this part of the experiments are as shown below.

As the first step of optimizing I used increasing doses of Nocodazol for 24 hrs. As results shown the highest percentage of the cells in G2/M is around 50%, which has been obtained with .125 ug/ul and .2 ug/ul and .3 ug/ul.

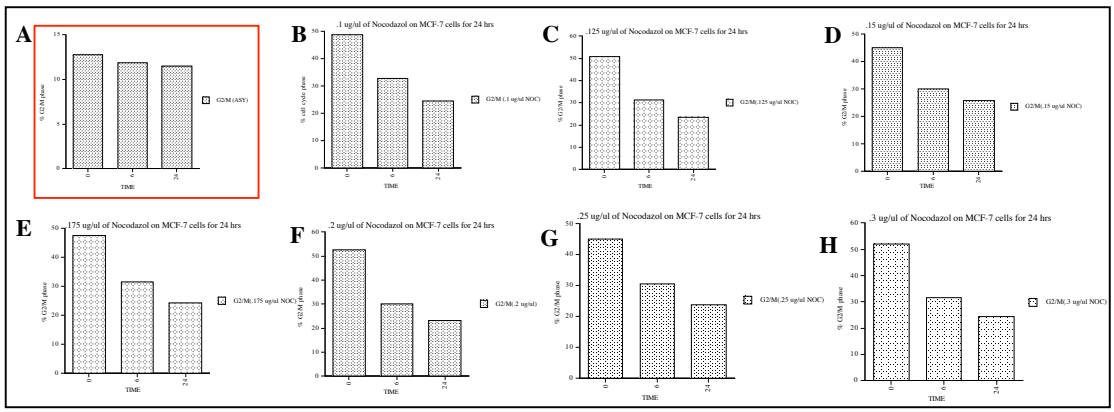


Figure 1- **G2/M phase of synchronized MCF-7 cells with Nocodazol for 24 hours. (Flow cytometric analysis).**
A) Asynchronous MCF-7 cells. B) MCF-7 cells synchronized with .1ug/ul of Nocodazol for 24 hours. C) MCF-7 cells synchronized with .125 ug/ul Nocodazol for 24 hours. D) MCF-7 cells synchronized with .15 ug/ul of Nocodazil for 24 hours. E) MCF-7 cells synchronized with .175 ug/ul of Nocodazol for 24 hours. F) MCF-7 cells synchronized with .2 ug/ul of Nocodazol for 24 hours. G) MCF-7 cells synchronized with .25 ug/ul of Nocodazol for 24 hours. H) MCF-7 cells synchronized with .3 ug/ul of Nocodazol for 24 hours.

As it has been shown in comparison to the asynchronous cells we got a good arrest of cells in G2/M phase but I wish to be able to synchronize higher percentage of the cells in G2/M phase. Since we are planning to look at the expression of ER-α in the G2/M phase so I think that having only 50% of the cells in G2/M and the other 50% in the other phases of the cell cycle could give us some misleading information about the expression of ER in the other phases of the cell cycle rather than only G2/M. So I decided to change the condition slightly in order to achieve a higher percentage in G2/M.
The next step we used the same increasing concentration of Nocodazol. However this time we left the Nocodazol on the cells for 36 hours. The reason for increasing the time of Nocodazol on the cells was to test the longer exposure to Nocodazol could yield a higher percentage of cells in G2/M phase. The result of this experiment reveals that 36 hours of Nocodazol on the cells is too much and causes the cells not being able to recover from G2/M.

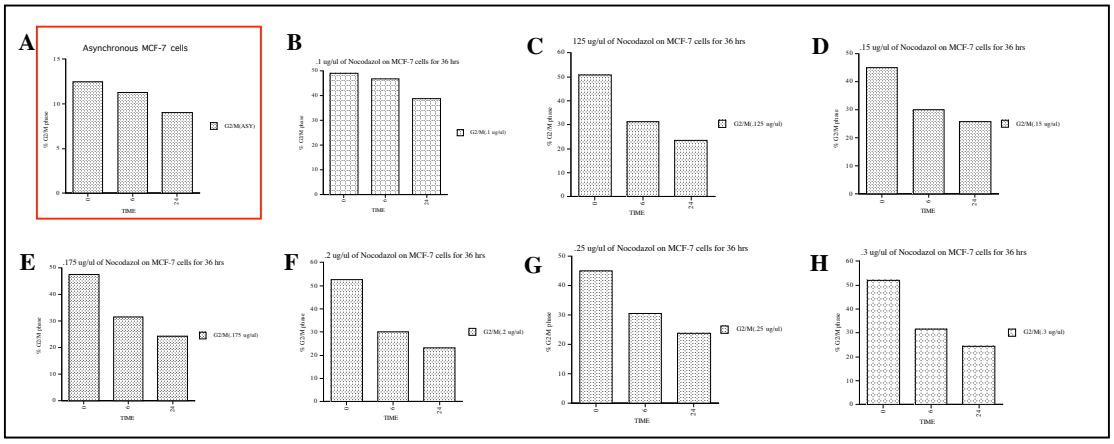


Figure 2- **G2/M phase of synchronized MCF-7 cells with Nocodazol for 36 hours. (Flow cytometric analysis).**
A) Asynchronous MCF-7 cells. B) MCF-7 cells synchronized with .1ug/ul of Nocodazol for 36 hours. C) MCF-7 cells synchronized with .125 ug/ul Nocodazol for 36 hours. D) MCF-7 cells synchronized with .15 ug/ul of Nocodazil for 36 hours. E) MCF-7 cells synchronized with .175 ug/ul of Nocodazol for 36 hours. F) MCF-7 cells synchronized with .2 ug/ul of Nocodazol for 36 hours. G) MCF-7 cells synchronized with .25 ug/ul of Nocodazol for 36 hours. H) MCF-7 cells synchronized with .3 ug/ul of Nocodazol for 36 hours.

As the next step we decided to do a sequential synchronization using the combination of Lovastatin and Nocodazol. Performing the sequential synchronization will allow the cells to accumulate in G1 phase by using Lovastatin and then release the cells from G1 phase using Mevalonate. As the cells flow in to S phase and then subsequently in to the

G2/M phase, we add Nocodazol in order to arrest a high percentage of cells, which are released from G1 phase in to the G2/M phase. The results of this experiment are depicted below.

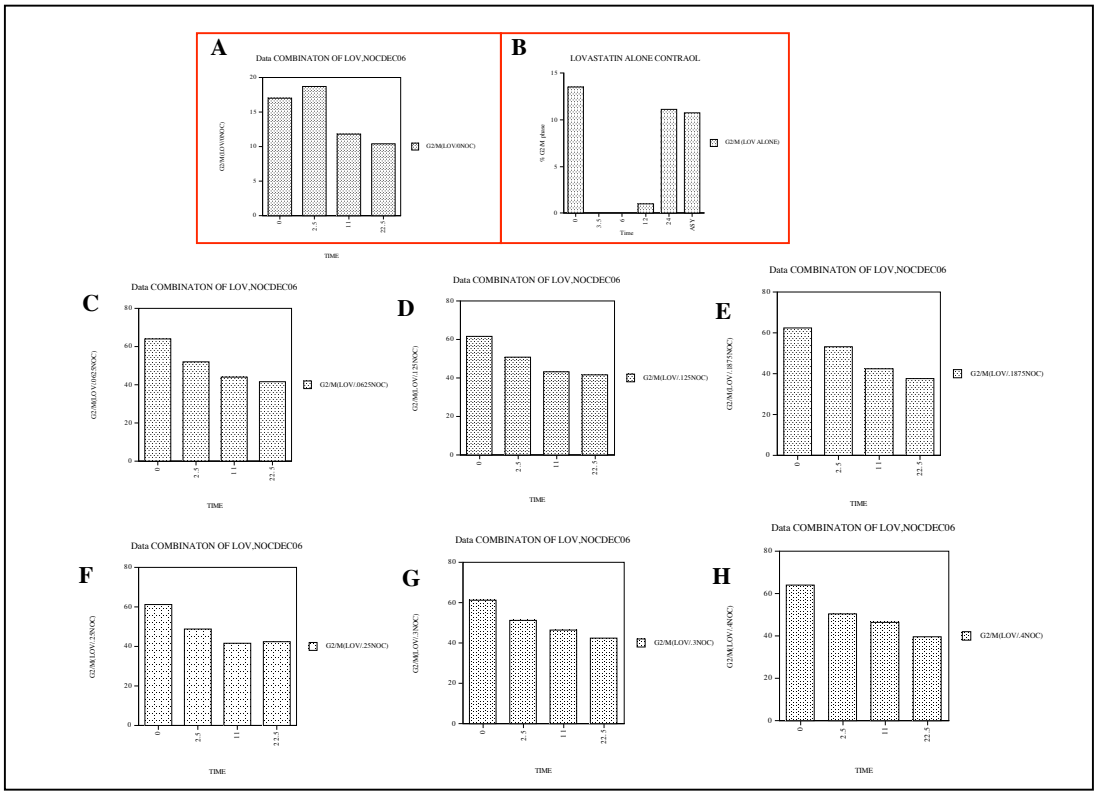


Figure 3- **G2/M phase of sequential synchronized MCF-7 cells with Lovastatin and Nocodazol. (Flow cytometric analysis).**

A) Asynchronous MCF-7 cells. B) MCF-7 cells synchronized with Lovastatin alone.
C) MCF-7 cells synchronized with Lovastatin and then with .0625 ug/ul of Nocodazol.
D) MCF-7 cells synchronized with Lovastatin and then with .125 ug/ul Nocodazol.
E) MCF-7 cells synchronized with Lovastatin and then with .1875 ug/ul of Nocodazil.
F) MCF-7 cells synchronized with Lovastatin and then with .25 ug/ul of Nocodazol.
G) MCF-7 cells synchronized with Lovastatin and then with .3 ug/ul of Nocodazol.
H) MCF-7 cells synchronized with Lovastatin and then with .4 ug/ul of Nocodazol.

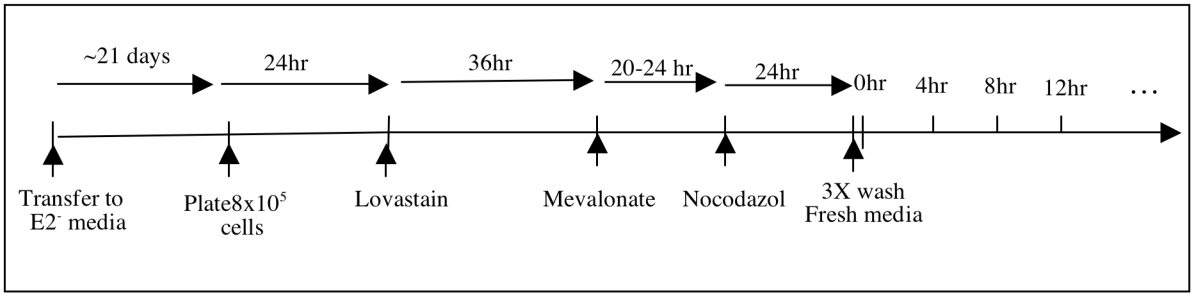
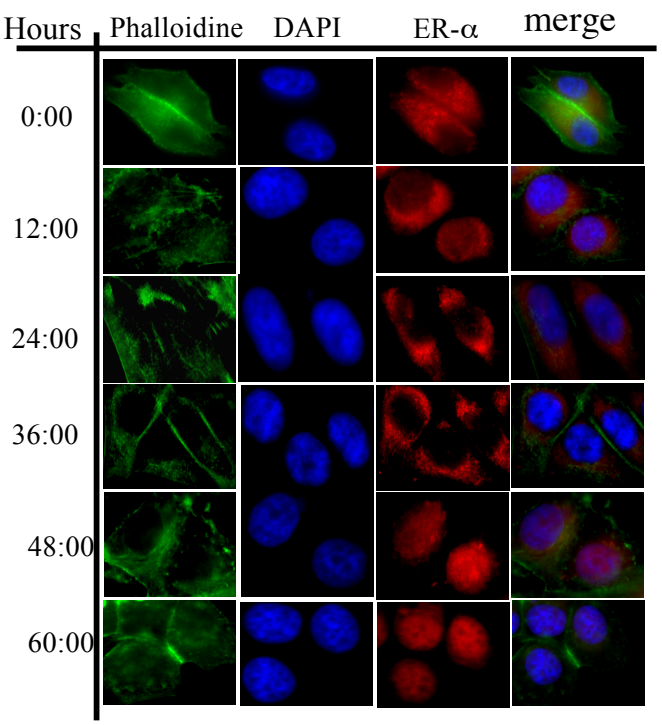


Figure 4- **Schedule of the sub sequential synchronization with Lovastatin and Nocodazol.**
MCF-7 Cells were synchronized with lovastatin for 36 hours in estrogen-free media and subsequently released from arrest with mevalonate. 20 to 24 hours after addition of Mevalonate different concentrations of Nocodazol were added to the cells. Nocodazol stayed on the cells for 24 hours. After 24 hours of Nocodazol treatment cells were washed three timen and subsequently fresh E2-free medium added to the cells. Cells were harvested at different time points after the release and processed for flow cytometry analysis.

c. Investigate the ER-α Localization in different cell cycle phases

Since ER-α expresses in a cell cycle dependent manner we would like to investigate the localization of the ER-α in different phases of the cell cycle. Cell cycle dependent translocation of ER-α could shed light on the mechanism of the cell cycle change that we have observed in our proposed study.
The cellular localization of ER-alpha is examined during cell cycle transition in MCF-7 cells by Immunofluorescence staining. . For immunofluorescent detection of ER-α MCF-7 cells cultured on coverslips, synchronized with lovastatin and stained with anti-ERα antibody. Cells stained to look at ER-α in the cell. The result of immunofluorescent study shows a translocation of ER-alpha from the cytoplasm to the nucleus during the progression of cell cycle from G1 to G2/M. ER-α localization is different in different phases of the cell cycle.

Figure 5- **Immunofluorescent staining of synchronized MCF-7 cells.**
Cells are plated on coverslips. Then cells are synchronized in G1 by lovastatin.
Cells were fixed and stained at different time points in order to look at the ER-α localization relative to the cell cycle phase.
Left panel is Phalloidine staining. Phalloidine is used to stain the cytoplasm in order to determine each cell's borders.
DAPI is used to stain the nucleus.
ER-α staining is in red.
Right panel is the merge of these stainings.



As it has been shown we have observed that ER is translocated from the cytoplasm to the nucleus upon exit from G1 phase of the cell cycle. We will further examine the role of ER translocation on its cell cycle regulation in synchronized population of cells. We will then examine the mechanism underlying the translocation of ER-α in different phases of the cell cycle through several different means. First we will correlate transcriptional activity of ER to its cellular localization at each phase of the cell cycle.

We will also examine the immuno-cpmplex formation of ER with other proteins as a strategy to identify the binding partners of ER under different ligand conditions we will use an in vitro (a combination of GST-fusion protein pull down assay and TNT® Quick Coupled Transcription/Translation System) and an ex vivo (mass spectrometry).

These experiments will help elucidate the mechanism by which cell cycle expression of ER alpha regulates the progression of cells through the cell cycle. Also these experiments will help us to reveal the binding partners of ER-α during the cell cycle, which, effects on different expression and function of ER-α during the cell cycle.

Key Research Accomplishments

- Illustration of the ER-α localization based on the cell cycle
- A combination method to synchronize most of the cells in G2/M phase of the cell cycle in a reversible manner.
- Construction of the adenoviral vectors to ER-α and GFP-ERα.

Reportable Outcomes

- Localization of ER-α is cell cycle dependent.

Conclusion and Future plans:

We could conclude that ER-α has different mechanism of cell cycle regulation in breast cancer. Additionally we can conclude that translocation of ER-α is a cell cycle dependent phenomenon in breast cancer cells.

- To finish the task 1 in my statement of work I will apply the best condition of the Nocodazol synchronization on the cells in order to show that the effect being described is not the effect of the arresting reagent.
- Also I will generate the virus containing ER-α and infect MDA-MB231 cells in order to test the effect of the exogenous ER-α on the cell cycle.
- Generation of the virus containing GFP-ERα will help me to look at the localization of the exogenous ER-α in the otherwise ER-negative cells.
- The last step for task 1 is to look at the partners of ER-α during the phases of the cell cycle. We will focus on the mechanism behind the partnership between the observed bound protein and ER-α in the specific cell cycle phase that has been observed.

The last step in task 1 will led us to start the task 2 of the proposed project.

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